

Activation of integrins in endothelial cells by fluid shear stress mediates Rho-dependent cytoskeletal alignment

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Fluid shear stress is a critical determinant of vascular remodeling and atherosclerosis. Both integrins and the small GTPase Rho are implicated in endothelial cell responses to shear but the mechanisms are poorly understood. We now show that shear stress rapidly stimulates conformational activation of integrin $\alpha v\beta 3$ in bovine aortic endothelial cells, followed by an increase in its binding to extracellular cell matrix (ECM) proteins. The shear-induced new integrin binding to ECM induces a transient inactivation of Rho similar to that seen when suspended cells are plated on ECM proteins. This transient inhibition is necessary for cytoskeletal alignment in the direction of flow. The results therefore define the role of integrins and Rho in a pathway leading to endothelial cell adaptation to flow.

Keywords: inside-out signaling/integrin affinity state/integrins/mechanotransduction

Introduction

Fluid shear stress due to blood flow is a major determinant of vascular remodeling, arterial tone and atherosclerosis (Davies *et al.*, 1997). The endothelial monolayer *in vivo* acts as a signal transduction interface for hemodynamic forces; these forces determine the shape, cytoskeletal organization and function of endothelial cells, allowing the vessels to cope with physiological or pathological conditions (Davies *et al.*, 1997). In regions of arteries where flow is disturbed and average shear stress is low, endothelial cells are polygonal and atherosclerosis develops (Caro *et al.*, 1969, 1971; Girard and Neren, 1995). In contrast, cells in regions of laminar shear elongate in the direction of flow and atherosclerosis is suppressed (Eskin *et al.*, 1984; Davies, 1991, 1995; Girard and Neren, 1993). Cell orientation and elongation are considered to be an adaptive process of endothelial cells to reduce the local mechanical load and subsequent injury. Despite the importance of mechanotransduction in vascular function and pathology, the molecular mechanisms by which endothelial cells sense and respond to fluid shear stress are not well understood.

A variety of data suggest a role for integrins in mechanotransduction. Endothelial cells must be anchored to their matrix in order to sense and transduce signals in response to shear stress (Takahashi and Berk, 1996). The attachment sites, termed focal adhesions, are complexes of integrins, cytoskeletal and signaling proteins that mediate signal transduction (Burridge and Chrzanowska-Wodnicka, 1996). A variety of published data implicate integrins in cellular responses to flow. Flow induces rapid remodeling of focal adhesion contacts, suggesting that these sites of cell attachment may be important in mechanotransduction (Davies *et al.*, 1993, 1994). The $\beta 1$ - and $\beta 3$ -containing integrins are predominant in cultured bovine aortic endothelial cells (BAECs) (Dejana, 1993); interestingly, integrin $\alpha v\beta 3$ is elevated in atherosclerotic plaques compared with quiescent regions of the same vessels (Hoshida *et al.*, 1995). *In vivo*, a peptide antagonist of integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ blocked flow-induced vasodilation (Muller *et al.*, 1997). It was shown further that the endothelial responses elicited by a $\beta 1$ -activating antibody resemble some of those induced by shear stress (Ishida *et al.*, 1996), and integrins transduce mechanical stimuli to biochemical signals via their association with Shc (Chen *et al.*, 1999). More recently, it was shown that integrins function as intermediates in a shear stress-induced signaling cascade to activate She and c-Jun NH₂-terminal kinases (JNKs) (Jalali *et al.*, 2001).

Rho, Rac and Cdc42 (Nobes and Hall, 1994) are closely related small GTP-binding proteins that have also been implicated in mediating cellular responses to mechanical stimuli. Rho has been proposed as a mediator of mechanical signaling (Yano *et al.*, 1996), based mainly on the ability of inhibitors of Rho to block cellular responses, including shear stress activation of JNK (Li *et al.*, 1999). These treatments, however, may have indirect effects by altering the actin cytoskeleton. Li and co-workers showed that shear stress induced the translocation of Rho from cytosol to membrane, which was taken to indicate activation (Li *et al.*, 1999). Membrane translocation of Rho family GTPases can, however, be regulated separately from activation (del Pozo *et al.*, 2000). There is therefore a need to assess more directly the roles of Rho family GTPases in mediating mechanical signaling.

Integrins and Rho GTPases are intimately connected at multiple levels (for review see Schwartz and Shattil, 2000). Several groups have described biochemical regulation of Rho activity by integrins. Cells plated on fibronectin (FN) showed an initial downregulation of Rho activity during the phase of active spreading, when the cytoskeleton is highly dynamic. As spreading reaches completion, Rho GTP loading is increased, reaching a maximum as focal adhesion and stress fibers are assem-

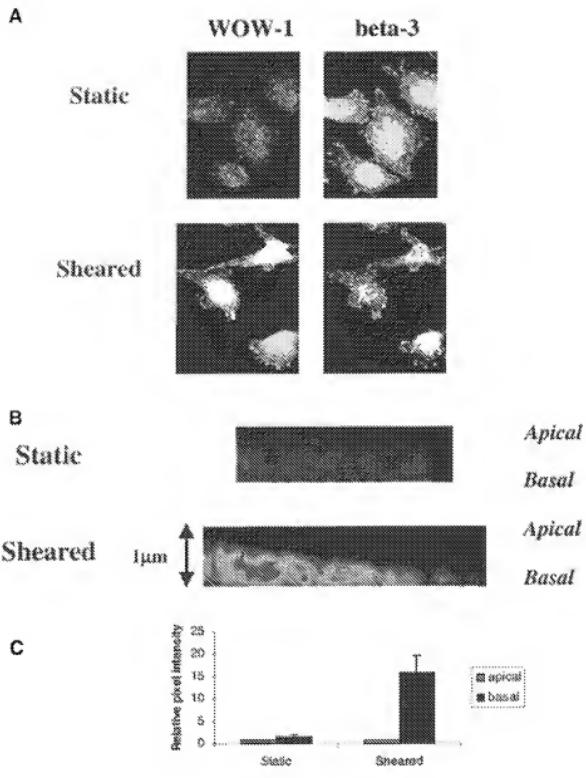


Fig. 1. Integrin $\alpha v\beta 3$ activation by shear stress. (A) BAECs under static conditions or after shear stress at 12 dynes/cm² for 5 min were fixed and stained with WOW-1 to label high-affinity unoccupied $\alpha v\beta 3$ and double labeled with a polyclonal antibody that recognizes all $\alpha v\beta 3$ integrins. (B) Color intensity profiles of representative XZ reconstructions of static and sheared cells that have been stained with WOW-1. Images show portions of single cells. The apical and basal surfaces are labeled. Red represents high and blue is low WOW-1 staining. (C) Histogram showing relative intensity of WOW-1 staining of basal compared with apical Z-sections of static and sheared cells. Cell edges were defined by TRITC-wheatgerm agglutinin (WGA) staining and were used as reference for quantitation of pixel intensity of WOW-1 staining; 45 cells were scored per condition.

bled, and then slowly returning to baseline (Ren *et al.*, 1999). These effects appear to be mediated by src-dependent phosphorylation of p190RhoGAP (Arthur *et al.*, 2000) and require focal adhesion kinase (FAK) (Ren *et al.*, 2000).

Despite numerous indications that integrins and Rho are in some way involved in mechanotransduction (Shyy and Chien, 1997; Chen *et al.*, 1999; Li *et al.*, 1999), the nature of their involvement is poorly understood. Jalali *et al.* (2001) found that shear stress induced an increase in endothelial monolayer staining with antibodies that

recognize the ligand-occupied conformations of $\beta 1$ and $\beta 3$ integrins. Additionally, blocking new integrin binding to extracellular cell matrix (ECM) proteins prevented the shear-induced association of integrins with the adapter protein Shc. These results suggested that dynamic integrin-ligand binding contributed to shear stress signaling. To delineate further this pathway, we have tested the role of integrin activation in the endothelial cell response to shear. We report that shear stress induces conformational activation of integrins followed by their increased binding to ECM ligands. This *de novo* occupancy then

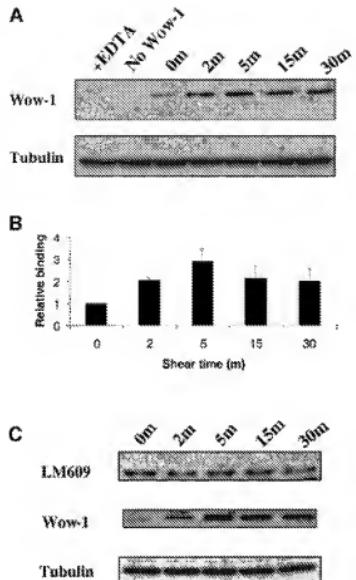


Fig. 2. Quantitation of high affinity $\alpha v \beta 3$ integrin. (A) BAEs were subjected to shear for the indicated times and incubated with WOW-1 to label activated $\alpha v \beta 3$. Bound antibody was quantitated by western blotting. As a control, EDTA was added to block specific binding. Blots were stripped and reprobed for tubulin to assess protein loading. (B) Histogram showing WOW-1 binding normalized to tubulin. Values are means \pm SEM ($n = 4$ independent experiments) relative to cells at time 0. Values are statistically significant as defined by a student's *t*-test ($P < 0.01$). (C) Coverslips were incubated with WOW-1 or LM609 and bound antibody detected by western blotting. Results are representative of three independent experiments. Blots were stripped and reprobed for tubulin to assess protein loading.

triggers a transient decrease in Rho activity that is required for cellular alignment.

Results

Shear stress increases high affinity $\alpha v \beta 3$

Application of fluid shear stress to resting BAEs induces integrin-ligand binding (Jalali *et al.*, 2001). This result suggests that shear stress may upregulate integrin function, consistent with many studies demonstrating that cell adhesion can be regulated by a variety of intracellular signaling pathways (Hato *et al.*, 1998). Integrin activation involves two complementary mechanisms, affinity and avidity modulation. Conformational changes in the integrin heterodimer lead to increased affinity for their ligands, whereas avidity modulation involves changes in lateral mobility and clustering of integrin heterodimers into oligomers that increase the binding of cells to

multivalent matrices. Few reagents are available to directly assess conformational integrin activation separate from effects on clustering/avidity. A recently developed engineered Fab fragment WOW-1 (Pampori *et al.*, 1999) reacts selectively with activated $\alpha v \beta 3$ integrin and provides a suitable assay for activation of this integrin. WOW-1 contains an Arg-Gly-Asp (RGD) tract and binds only to unoccupied integrins in a ligand-mimetic fashion. Since WOW-1 is monovalent, it is relatively insensitive to changes in integrin clustering and reports mainly changes in integrin affinity.

To test whether shear stress altered activation of $\alpha v \beta 3$ integrin, confluent monolayers of BAEs were subjected to an increase in shear stress from 0 to 12 dynes/cm². Cells were then fixed, labeled with fragments of WOW-1 and counterstained with a polyclonal antiserum against the $\beta 3$ integrin subunit to label the total pool of $\alpha v \beta 3$. The majority of unsheared cells showed only weak WOW-1 staining, whereas the anti- $\beta 3$ antibody stained both focal adhesions and diffusely over the cell surface as expected (Figure 1A). Shear stress caused the formation of lamellipodia, which correlated with increased WOW-1 staining, while total $\alpha v \beta 3$ appeared unchanged. Some WOW-1 staining at the interface of neighboring cells was also observed (not shown). As a control, cells were labeled with WOW-1 in the presence of EDTA, which blocks specific ligand binding to $\alpha v \beta 3$ and this showed minimal staining (not shown but see Figure 2A).

To determine whether the active integrin was localized to the basal or apical surface, samples were analyzed using laser scanning confocal microscopy. Endothelial cells are very flat ($\sim 1 \mu\text{m}$ thick), which is sufficiently close to the limits of resolution for optical microscopy ($\sim 0.4 \mu\text{m}$) that the upper and lower surfaces cannot be fully resolved. However, examination of XZ reconstructions of the Z-sections showed low levels of WOW-1 staining on both apical and basal surfaces in static endothelial cells, whereas cells exposed to shear showed an increase in high affinity $\alpha v \beta 3$ primarily in the basal sections (Figure 1B). Quantification of staining intensity at the upper and lower surfaces (Figure 1C) showed that WOW-1 staining increased dramatically in the basal surface while barely changing at the apical surface. These results indicate that the shear-induced increase in activated $\alpha v \beta 3$ is primarily basal in location. Previous studies using the anti- $\alpha v \beta 3$ integrin antibody LM609 showed that shearing caused an enhanced staining at the peripheral region of the abluminal side of the cells and no change in the luminal side (Li *et al.*, 1997), in agreement with our findings.

To quantitate total WOW-1 staining, cells were incubated with WOW-1 and rinsed; lysates were prepared and the amount of bound WOW-1 was determined by western blotting (Figure 2A). These results showed that shear stress stimulated an increase in high affinity $\alpha v \beta 3$ that was approximately half maximal by 2 min; binding reached a maximum at 5 min that was ~ 3 -fold relative to baseline ($P < 0.01$ compared with static control), then declined to ~ 2 -fold at 30 min (Figure 2B). EDTA abolished binding of WOW-1 to sheared cells, demonstrating specificity. LM609, an $\alpha v \beta 3$ -specific antibody that is insensitive to integrin activation state, showed no increase in binding to sheared cells compared with static controls (Figure 2C). This finding indicates that the increase in WOW-1 binding

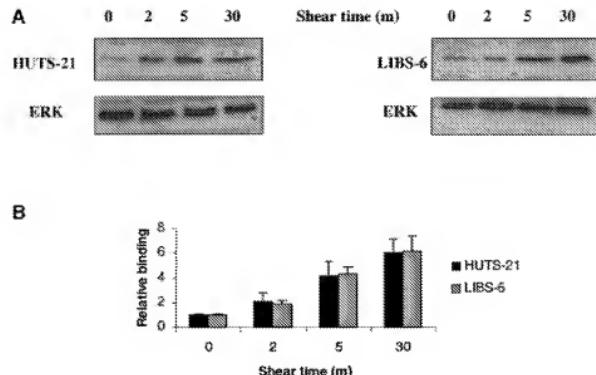


Fig. 3. Quantitation of integrin occupancy. (A) BAEs plated on VN or FN were sheared for the indicated times and incubated with 5 μ g/ml LIBS-6 or HUTS-21, respectively. Bound antibody was assessed by western blotting. Blots were stripped and reprobed for extracellular signal regulator kinase (ERK) to assess protein loading. (B) Results from three independent experiments were quantitated. Values are means \pm SEM.

after shear stress represents a specific increase in the high affinity $\alpha\beta 3$ integrins, rather than a general increase in surface expression. WOW-1 is ligand mimetic and can therefore bind only unoccupied integrins (Pampori *et al.*, 1999). The increase in WOW-1 staining shortly after application of shear stress and the subsequent decrease therefore suggests that the high-affinity unoccupied $\alpha\beta 3$ may rapidly bind to immobilized ECM, which would then prevent WOW-1 binding.

Shear stress stimulation of integrin binding to ECM

Affinity modulation as monitored by WOW-1 binding could induce integrin binding to ECM. In order to quantitate the increase in integrin-ligand binding in response to shear stress we used two markers for ligand-bound integrins. The ligand-induced binding site antibodies LIBS-6 and HUTS-21 have high affinities for ligand-occupied $\beta 3$ (Hughes *et al.*, 1997) and $\beta 1$ integrins (Luque *et al.*, 1996; Gómez *et al.*, 1997), respectively. These antibodies also stabilize the ligand-occupied conformation of integrins and can therefore activate them, but at the low concentrations and short times used in these experiments they would serve primarily as a read-out for integrin without strongly perturbing integrin activation state.

It is well established that cells on FN and vitronectin (VN) for 2 h assemble a matrix and make focal adhesions similar to the endogenous matrix produced by cells cultured overnight (Hayman and Ruoslahti, 1979; Avnur and Geiger, 1981; Dejana *et al.*, 1988). BAEs were sheared for the indicated times and the binding of the antibodies was assessed by western blotting. As predicted, shear stress increased LIBS-6 binding to BAEs that had been plated on VN, and HUTS-21 binding to cells on FN (Figure 3). Binding of LIBS-6 and HUTS-21 was detectable at 2 min but was $<20\%$ of maximal (Figure 3).

In contrast to WOW-1, the binding of LIBS-6 and HUTS-21 continued to increase at later times of shear stress. To verify that increased staining with these antibodies

indicates ligand binding, cells plated on FN or VN were stained with both reagents. Cells on VN showed marked LIBS-6 binding but no increase in HUTS-21 binding, consistent with the specificity of VN for $\alpha\beta 1$ integrins. Conversely, cells on FN showed a large increase in HUTS-21 binding but only a slight increase in LIBS-6 binding, again consistent with the preference of FN for $\alpha\beta 1$ relative to $\alpha\beta 3$ (not shown). These observations, together with the WOW-1 binding time courses, suggest that integrin activation is followed temporally by the formation of integrin-ligand complexes.

Biphasic regulation of Rho activity during shear stress

New integrin-ligand binding would be predicted to induce many of the same signals seen in cells freshly plated on ECM proteins. As Rho has been suggested to play an important role in endothelial cell responses to shear, we assayed Rho GTP loading using a recently developed pull-down assay (Ren *et al.*, 1999). BAEs were sheared for the indicated times and Rho-GTP loading assays were performed. Following a lag period of ~ 2 min, shear triggered a decrease in Rho activity at 5–15 min that was followed by an increase that peaked at 60 min, which was statistically significant ($P < 0.05$) (Figure 4B).

In order to correlate the effect of shear stress on Rho activity with its known actions on the cytoskeleton, sheared cells were fixed and stained with rhodamine-phalloidin to visualize F-actin. In sheared cells, the initial phase of low Rho activity is associated with a decrease in stress fibers, while the later restoration of Rho activity corresponds to an increase in stress fibers (Figure 4C). Reappearance of stress fibers at 60 min is accompanied by

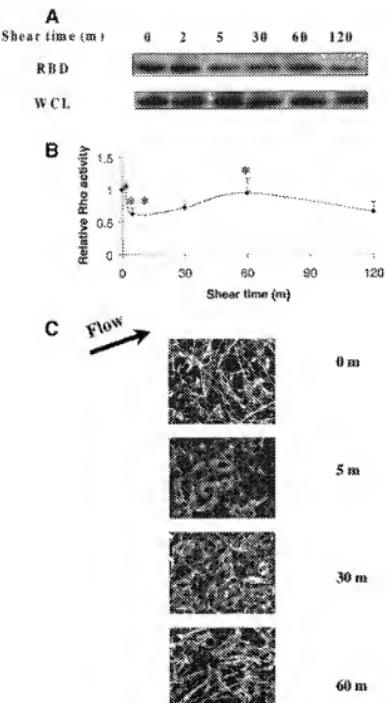


Fig. 4. Regulation of Rho activity by shear stress. (A) BAEs were subjected to shear stress for the indicated times. Rho activity is indicated by the amount of RBD-bound Rho, normalized to total Rho in whole cell lysates (WCL). (B) Quantitation of Rho activity relative to cells at time 0. Values are means \pm SEM from three independent experiments, each of which was performed in duplicate. For the statistical analysis 5 min was compared with 0 min (* $P < 0.01$) and 60 min was compared with 5 min (** $P < 0.05$). (C) Time course of F-actin organization. Cells were subjected to shear stress, fixed and stained with rhodamine-phalloidin to detect actin stress fibers.

significant cell alignment in the direction of flow, although maximal alignment requires longer times of exposure to flow (Levesque and Neren, 1985). Rho activity decreases again at 120 min, although actin stress fibers remain. We do not fully understand this result, although very similar effects were seen during adhesion to FN (Ren *et al.*, 1999). It may be that Rho is highest during assembly of stress fibers and that lower levels are sufficient for their maintenance.

New ECM binding mediates stress-induced Rho activation

The response in Rho activity to shear is highly reminiscent of the effect of plating cells on FN (Ren *et al.*, 1999;

Arthur *et al.*, 2000). Comparison of time courses of integrin-ligand binding (Figure 3) with that of Rho activation (Figure 4) in response to shear stress reveals that binding of integrins to ECM occurs slightly before Rho inhibition. We therefore tested whether the change in Rho activity requires new integrin binding to ECM by preventing formation of new integrin-ligand connections without affecting the existing adhesions. Endothelial cells were plated on FN for 2 h to allow completion of cell spreading and formation of adhesions. To study the effects of blocking the unoccupied FN sites, the cells were treated for 15 min with either blocking (16G3) or non-blocking (11E5) anti-FN antibodies. Shear stress was then applied to the cells and Rho assays were performed. No effect on cell shape or actin staining patterns could be detected after this treatment (not shown), indicating that the antibodies did not disrupt existing integrin-ligand bonds (not shown). Rho assays showed that the blocking antibody 16G3 strongly inhibited the shear stress-induced decrease in Rho activity, whereas the non-blocking antibody 11E5 had no effect (Figure 5). These results provide support for the hypothesis that regulation of Rho activity by shear stress is downstream of new integrin-ligand binding.

To test whether integrin activation is sufficient to modulate Rho activity, its activity was assayed following integrin activation by two procedures. First, BAEs were treated with the activating anti- $\alpha v \beta 3$ antibody LIBS-6 (Huang *et al.*, 1993) at a concentration previously shown to activate $\alpha v \beta 3$ integrin (Frelinger *et al.*, 1990; O'Toole *et al.*, 1994). Secondly, $MnCl_2$ was added to activate all of the integrins. Although we did not observe changes in morphology, these treatments increase integrin binding to ECM and adhesion strength (Palecek *et al.*, 1997; García *et al.*, 1998). Both LIBS-6 and $MnCl_2$ caused dramatic decreases in the activity of Rho, which showed partial recovery at 60 min (Figure 6). The regulation of Rho activity by artificial activation of integrins is similar to but more pronounced than that seen after the mechanical activation by shear stress (Figure 4). These results show that increased integrin activation in response to shear stress can account for the transient inhibition of Rho.

Downregulation of Rho is essential for the shear stress-induced cell alignment

Exposure of endothelial cells to shear stress results in their elongation along the direction of flow (Levesque and Neren, 1985), the formation of stress fibers and alignment of microfilaments (Girard and Neren, 1993). We hypothesized that the shear stress-induced decrease in Rho activity may be required for this response. To test this idea, BAEs were transiently transfected with expression vectors for constitutively activated Rho (V14Rho) together with green fluorescent protein (GFP) as a marker for transfected cells. Alternatively, cells received wild-type (WT) Rho or empty vector as controls. The cells were then sheared for 16 h, fixed and stained for actin. GFP-positive cells were then examined for elongation and orientation with the direction of fluid flow. As shown in Figure 7, few of the static cells were aligned. After 16 h of shear, BAEs expressing GFP alone or WT Rho aligned with the direction of flow and formed stress fibers similarly to untransfected cells. In contrast, expression of constitutively activated V14Rho strongly inhibited cell and

cytoskeletal alignment in response to shear stress. These results demonstrate that the initial decline in Rho activity is required for cells to orient with the direction of flow at later times.

Discussion

In the present study, we have investigated the roles of integrins and Rho in endothelial shear stress signaling. Previous studies have established that endothelial cells respond to physiological levels of shear stress in the following manner: cells initially enhance their attachments to the ECM and neighboring cells, followed by a loss of the dense peripheral bands and an increase in motile

behavior. Cells subsequently increase their stress fibers as cell alignment and elongation begin (Galbraith *et al.*, 1998). This adaptation to flow is believed to contribute to a reduction of shear gradients along the endothelial cell surface (Barbee *et al.*, 1994). It is possible that the shear stress-induced cytoskeletal changes are facilitated by integrin signaling. For example, shear stress causes activation of FAK (Li *et al.*, 1997) and remodeling of focal adhesions in the direction of flow within several minutes (Davies *et al.*, 1994). Indeed, the dynamic remodeling of the adhesion plaques in endothelial cells exposed to shear stress requires constant association and dissociation of integrins with the ECM (Davies *et al.*, 1994). One of the general mechanisms for regulation of integrin function involves their activation. As a consequence of such activation, integrins enhance their apparent affinity or avidity for their extracellular ligands (Bazzoni and Hemler, 1998).

In this study, we show for the first time that integrin $\alpha v\beta 3$ is activated by shear stress. Activation appears to be the result of affinity modulation because sheared cells show increased binding to a conformation-sensitive anti- $\alpha v\beta 3$ antibody, WOW-1 Fab. Integrin $\alpha 5\beta 1$ also shows increased binding to ECM following shear stress (Jalali *et al.*, 2001); although no specific probe is available to assess $\alpha 5\beta 1$ activity, it seems likely that it too is activated by shear stress. Analysis of the immunofluorescence data suggested that shear-induced increase of high affinity $\alpha v\beta 3$ integrin was primarily on the basal surface of BAEs. Davies *et al.* (1994) showed that focal adhesion sites at the abluminal endothelial membrane are both acutely and chronically responsive to frictional shear stress forces applied to the opposite (luminal) cell surface. Our previous report that shear stress increases integrin-ligand binding on the basal surface of human umbilical vein endothelial cells (HUVECs) (Jalali *et al.*, 2001) is in concert with the hypothesis that integrin activation is followed by the formation of integrin-ligand complexes on the basal surface of endothelial cells. However, the

Fig. 5. Blocking free FN sites. BAEs were plated on FN-coated slides for 2 h in the absence of serum and then incubated with anti-FN Fab fragments for 15 min. Cells were either sheared (5 min, 12 dyne/cm²) or kept as static control and Rho assayed. Values are means \pm SEM ($n = 3$, each in duplicate) relative to static samples.

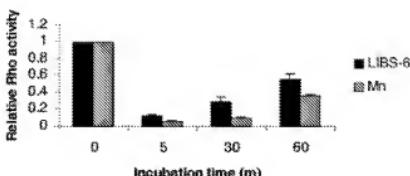
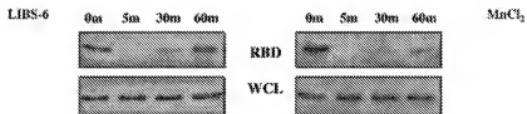
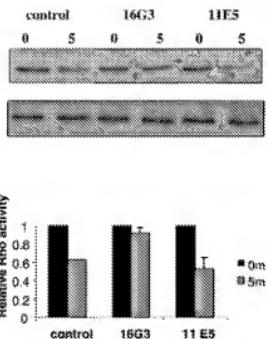


Fig. 6. Integrin activation mimics the shear-induced Rho activation. BAEs were incubated with 0.5 mM MnCl₂ or 15 μ g/ml LIBS-6 antibody for the indicated times and Rho activity was determined. Values are means \pm SEM ($n = 3$, each in duplicate) and represent Rho activity relative to cells at time 0.

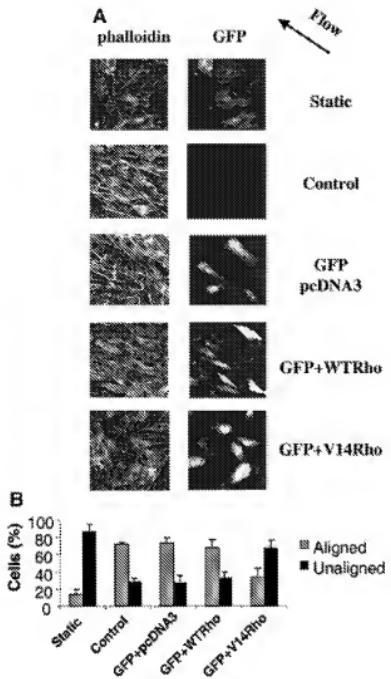


Fig. 7. Constitutively activated Rho blocks cell alignment. (A) Cells transiently transfected with empty vector, WT Rho or V14Rho plus GFP were subjected to shear stress or kept under static conditions for 16 h. The cells were fixed, stained with rhodamine-phalloidin and observed by confocal microscopy. The direction of flow is indicated by an arrow. (B) Quantification of cell alignment with the direction of flow. Values are means \pm SEM, $n = 3$; >100 cells were scored per condition.

restriction of active $\alpha\beta 3$ to the basal surface may be a general feature of endothelial cell behavior rather than a specific consequence of shear stress signaling. Active integrins on the luminal surface of endothelia would be highly thrombogenic; thus, these cells may have evolved mechanisms to suppress high affinity integrins on the luminal side. Whether this spatial specificity is a feature of a cell's response to shear stress or a general aspect of endothelial cells is therefore unclear.

We have shown that shear stress regulates Rho activity by performing GTP loading assays. These results implicate Rho as an important regulator of the shear-induced cytoskeletal reorganization. We suggest that the observed regulatory effects on Rho activity closely match the requirements for shear stress-induced cell alignment. Either inhibiting Rho by treatment of cells with C3 toxin

or transfection with a dominant-negative form of Rho (Li *et al.*, 1999) or constitutively activating Rho decreases cell alignment, just as it decreases cell migration (Takaiishi *et al.*, 1993; Allen *et al.*, 1998; del Pozo *et al.*, 1999). Thus, dynamic regulation of Rho is important for cell alignment. Importantly, our observations place Rho at a site downstream of integrins binding to their immobilized ligands. These findings are in agreement with those described in the study of Jalali *et al.* (2001), which showed that new connections between integrins and matrix proteins were needed for integrins to associate with Shc and activate JNK in response to shear stress.

The molecular mechanism(s) responsible for the cross-talk between integrins and the initial downregulation of Rho activity in response to shear stress are now becoming clearer. We have observed that fibroblasts from FAK^{-/-} mice failed to transiently inhibit Rho activity when plated on FN, whereas re-expression of FAK restored normal Rho regulation (Ren *et al.*, 2000). Another report has focused on the role of c-Src-mediated integrin signaling in modulating RhoA activity during cell adhesion through tyrosine phosphorylation of p190RhoGAP GTPase-activating protein (Arthur *et al.*, 2000). The same investigators also demonstrated the existence of a protein tyrosine phosphatase Shp-2, sensitive to calpeptin, acting upstream of RhoA (Schoenwaelder *et al.*, 2000). Future studies aiming to determine the signaling events that activate Rho following its transient inhibition by shear stress are of particular interest in elucidating fundamental mechanisms in mechanotransduction.

In summary, the work presented here sheds new light on the mechanism by which integrins mediate mechanotransduction in response to shear stress. We have defined a pathway in which conversion of the integrin $\alpha\beta 3$ to its high affinity state leads to new integrin binding to the ECM. These new integrin-ECM connections generate a signaling cascade similar to cell adhesion and are essential for the downstream signaling to Rho, which allows endothelial cell alignment as a mechanism of adaptation to hemodynamic forces.

Materials and methods

Cell culture and shear stress

BAECs were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine (Gibco-BRL, Gaithersburg, MD). All cell cultures were kept in a humidified 5% CO₂/95% air incubator at 37°C. BAECs cultured on 38 \times 76 mm slides to confluence were either kept as static controls or subjected to shear stress in a parallel plate flow chamber (Frangos *et al.*, 1985) modified so that multiple slides could be sheared simultaneously. A surface area of 14 cm² on the BAEC-seeded slide was exposed to fluid shear stress generated by perfusing culture medium over the cells. The pH of the system was kept constant by gassing with 5% CO₂/95% air and the temperature was maintained at 37°C. Shear stress at 12 dynes/cm² was used for all experiments, which is relevant to the physiological range in human arteries.

Detection of activated integrins

BAECs grown on FN-coated slides were starved overnight in 0.5% serum then shear stress applied. Where indicated, cells were treated with 1 mM MnCl₂ to activate all integrins or 15 μ g/ml LIBS-6 (a gift from Dr M.H. Ginsberg) to activate $\beta 3$ integrins, as previously described (Freling *et al.*, 1990; O'Toole *et al.*, 1994). Coverslips were incubated with His₆-tagged recombinant WOW-1 Fab (30 μ g/ml) or LM609 (15 μ g/ml) (a gift from Dr D.A. Cheresh) for 30 min, washed in phosphate-buffered saline (PBS) and lysed in SDS sample buffer. Bound

WOW-1 was detected by western blotting using a goat anti-rabbit antibody against His (Santa Cruz Biotechnology) and horseradish peroxidase (HRP)-protein A (Amersham Pharmacia Biotech).

For detection of occupied integrins, BAECs plated on FN- and VN-coated slides were starved overnight in 0.5% serum prior to application of shear stress for the indicated times. Cells were incubated with LIBS-6 (5 µg/ml) or HUTS-21 (5 µg/ml) (a gift from Drs F.Sánchez-Madrid and C.Cabauas) for 20 min at 37°C. Cells were rinsed with PBS, lysed in SDS sample buffer and bound IgG detected by western blotting using an HRP-goat anti-mouse antibody.

GPtase assays

BAECs were seeded on slides coated with FN (5 µg/cm²), grown to confluence and starved overnight in 0.5% serum. After shear stress, cells were chilled on ice, washed with ice-cold PBS and lysed in RIPA buffer [50 mM Tris pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂, 10 µg/ml each of leupeptin and aprotinin, 1 mM phenylmethyl sulfonyl fluoride (PMSF)] (Ren et al., 1999). Lysates were centrifuged at 13 000 g at 4°C for 10 min, and equal volumes were incubated with glutathione S-transferase-RBD (GST-RBD) beads (20 µg of protein per sample) at 4°C for 45 min. The beads were washed with buffer containing 50 mM Tris pH 7, 0.5% NP40, 500 mM NaCl, 1 mM MgCl₂, 1 mM EGTA and 10 µg/ml each of leupeptin and aprotinin, 1 mM PMSF. Rho was detected by western blotting using a polyclonal antibody against RhoA (Santa Cruz Biotechnology).

Blocking of unoccupied FN

BAECs were serum starved overnight in 0.5% serum-containing media, plated on FN-coated glass slides and allowed to adhere for 2 h. The cells were incubated with 20 µg/ml Fab fragments of anti-FN monoclonal antibodies for 15 min, then shear was applied for 5 min. Fab fragments were produced as previously described (Harlow and Lane, 1988). The antibodies were: 16G3, which blocks both the αvβ3 and α5β1 binding sites for FN, and 11E5, which is a non-function blocking antibody (a generous gift from Dr K.M.Yamada).

Fluorescence microscopy

Cells were fixed for 30 min in 2% formaldehyde in PBS, permeabilized in 0.2% Triton X-100/PBS, and rinsed twice with PBS. Non-specific sites were blocked with 10% goat serum, incubated with WOW-1 (30 µg/ml) and stained with 10 µg/ml Alexa green-conjugated goat anti-mouse Fab'2 (Molecular Probes). They were also labeled with a polyclonal β3 serum (a gift of Dr Mark Ginsberg, Scripps Research Institute) at 1:100, followed by 1:100 CY5-conjugated goat anti-rabbit Fab'2 fragment (Sigma) and mounted in immunofluorescence mounting medium (ICN Immunobiologicals). Where indicated, cells were stained with TRITC-phalloidin (Sigma) or TRITC-wheatgerm agglutinin (WGA) (Sigma). Confocal serial sectioned images were acquired using a BioRad 1024 MRC scanning confocal microscope. Serially sectioned composite reconstructions were used to obtain a Z-section through the longitudinal plane of each cell. The pixel intensity profile of each image, comparing the apical and basal surfaces, was analyzed using Image Pro Software.

V14Rho transfections

BAECs were seeded on FN-coated slides and at ~50% confluence were transfected with 2.25 µg of hemagglutinin-tagged V14 Rho or WT Rho in pcDNA3 plus 0.25 µg of vector encoding enhanced GFP using Effectene reagents (Qiagen), according to the manufacturer's instructions. After 10 h in growth medium cells were starved overnight in 0.5% serum. They were then subjected to shear stress for 16 h, fixed and stained with TRITC-phalloidin.

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